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(54) Title: OLIGONUCLEOTIDES THAT BLOCK TOLL-LIKE RECEPTORS

(57) Abstract: Compositions and methods are provided for inhibiting the expression of Toll-like receptor-4 genes. Antisense and dsRNA oligonucleotides targeted to nucleic acids encoding toll-like-receptor-4 are preferred. Methods of using these oligonucleotides for inhibition of toll-like-receptor-4 expression, and thereby preventing the signaling of toll-like receptor-4 ligands and for treatment of diseases including septic shock, inflammatory and autoimmune diseases associated with toll-like receptor-4 ligands, are provided.



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OLIGONUCLEOTIDES THAT BLOCK TOLL-LIKE RECEPTORS

FIELD OF THE INVENTION

5 The present invention relates to novel oligonucleotide sequences, capable of inhibiting the expression of Toll-like receptor- 4. The present invention further relates to pharmaceutical compositions comprising such oligonucleotides and to methods for using such compositions for the prevention or treatment of septic shock in gram-negative bacterial infections and for prevention or treatment of inflammatory and autoimmune diseases in which heat shock protein 60 (Hsp60) or other ligands of Toll like receptors are
10 involved.

BACKGROUND OF THE INVENTION

Toll proteins

15 The Toll-like receptors (TLRs) are proteins that contribute to the signal transduction induced by many pathogen-associated molecular patterns , and perhaps also to endogenous damage signals generated at sites of inflammation.

An isolated and purified DNA molecule comprising a human genomic DNA segment encoding Toll-like receptor 4 and a biologically active fragment or variant thereof are disclosed and claimed in PCT publication WO 00/77204.

20 International application WO 99/20756 discloses DNA sequences encoding human Toll polypeptides and antibodies which specifically bind these polypeptides. The polypeptides are used to identify other proteins involved in Toll-mediated transduction (e.g. natural ligands), to screen for receptor and ligand mimics, and to generate antibodies.

25 International application WO 98/50547 discloses human DNA of Toll-like receptor proteins and peptides derived from them. The compounds are claimed for use in altering phosphate metabolism, modulating inflammatory function or innate immunity responses. A binding compound, preferably an antibody or antibody fragment which specifically binds to these proteins or peptides, is also disclosed. However, the exact role of the Toll proteins in the above-mentioned processes has not been established.

Toll-like-receptor 4 and hsp60

The previously described ligands for Toll-like receptors in mammalian cells are of microbial origin, which is in line with a function of these receptors in innate immune responses. It was recently found that the chaperone hsp60 is a putative endogenous ligand of Toll-like receptors in mammals (Ohashi et al., J Immunol. 164:558-561, 2000). This finding suggests that Toll-like receptors may not only have a function in innate immune defense against microbial pathogens but also have a major physiological role in interacting with endogenous ligands.

It is noteworthy that both Toll-like receptors and hsp60 are found early in phylogeny and both are of remarkably conserved structure. This indicates that their interaction is relevant and may also occur in more primitive organisms. This is reminiscent of the situation in *Drosophila* where Toll controls dorsal-ventral patterning with spaetzle serving as endogenous ligand (Marisato and Anderson, Cell 76: 677-688, 1994), while in adult insects Toll controls the antifungal and antibacterial response (Lemaitre et al., Cell 86: 973-983, 1996).

Several studies revealed that mice resistant to endotoxic shock carry natural mutations in Tlr4 (Qureshi S. et al., J. Exp. Med 189:615-625, 1999). In humans, a number of polymorphic alleles of Tlr4 have been identified. One such relatively common Tlr4 polymorphism may be associated with an increased risk of septic shock and a decreased risk of atherosclerosis, but is not associated with increased risk of meningococcal disease.

Mammalian hsp60 usually is sequestered to the cell interior, in accordance with its ability to function as chaperone. However, hsp60 becomes accessible when it is set free during necrosis of tissue cells during inflammation or when hsp60 is partially translocated to the plasma membrane in response to diverse types of stress. It was therefore proposed that autologous Hsp60 may serve as a danger antigen to the innate immune system (Chen et al. J Immunol. 162:3212-3219, 1999).

In addition to LPS, self and foreign heat shock protein 60 (Hsp60) activate cells in a Tlr4 dependent manner (Ohashi et al., J Immunol. 164:558-561, 2000). Hsp60 has been implicated in multiple autoimmune and inflammatory conditions including but not limited to juvenile rheumatoid arthritis, type 1 diabetes, multiple sclerosis, systemic lupus erythematosus, inflammatory bowel disease, Bechet's disease, uveitis, thyroiditis, and atherosclerosis (Cohen IR., Annu Rev Immunol. 9:567-589, 1991; Jaattela M., Ann Med.

24:249-258, 1992). In many of these conditions, Hsp60 is present in a soluble form in the circulation and anti-Hsp60 antibodies and T cells can be detected in high titer (Xu et al., Circulation 102:14-20, 2000). Innate immune cell activation by LPS has also been implicated in some of these conditions (Balasa et al., Clin Immunol. 95:93-98, 2000).

- 5 International application WO 01/43691 discloses fragments and antagonists of Hsp60 capable of inhibiting the Tlr4, for treatment of inflammatory and autoimmune diseases.

Hsp60 and Tlr4 in Inflammatory and Autoimmune conditions

- Self and foreign Hsp60 activate macrophages in a Tlr4 dependent manner.
- 10 Circulating Hsp60 and/or anti-Hsp60 antibodies and T cells can be found in high titer in multiple autoimmune and inflammatory conditions such as rheumatoid arthritis, type 1 diabetes, and atherosclerosis. Hsp60 activation of innate immune cells as well as endothelial and muscle cells through Tlr4 may contribute significantly to the pathogenesis of these conditions. Thus, there is a need to modulate the role of autologous hsp60 and
- 15 possibly of Toll-like receptor complexes involved in certain diseases and in particular in the regulation of pro-inflammatory immune responses and to provide molecules which are useful to suppress or even prevent such responses.

Septic Shock

- 20 Systemic gram-negative bacterial infections can cause multi-organ failure and vascular collapse, a syndrome referred to as septic shock. Despite aggressive antibiotic therapy and full supportive care provided in intensive care units, septic shock carries a high morbidity and mortality. Lipopolysaccharide (LPS), also referred to as endotoxin, is responsible for many of the events leading to septic shock. LPS signals through a surface
- 25 receptor complex comprising CD14 and Toll-like receptor 4 (Tlr4) that is present on multiple cell types (Chow et al., J Biol Chem. 274(16):10689-10692, 1999).

- LPS activation through Tlr4 leads to release of large amounts of pro-inflammatory cytokines such as Tumor necrosis factor- α (TNF α). Therapeutic strategies targeting the cytokine response to LPS are under investigation in clinical trials for septic shock. A recent
- 30 large clinical trial showed a small but significant benefit in treating patients with a TNF α monoclonal blocking antibody, thereby reducing mortality. However, many studies using anti-TNF α antibodies and soluble TNF α receptor proteins have shown no significant

decrease in mortality (Reinhart K et al., Crit Care Med (7):S121-125, 2001). Nevertheless, the current paradigm in gram-negative septic shock treatment combines antibiotic therapy, supportive measures, and suppression of the overwhelming cytokine response to LPS.

A number of publications (including for example US 6,228,642, WO 93/09813, EP 0 414 607B1 and WO 95/00103) disclose the use of antisense oligonucleotides targeting nucleic acids encoding TNF α . Several groups are developing antisense oligonucleotides for the treatment of septic shock targeting either the TNF α gene (Ponnappa et al., J Pharmacol Exp Ther. (3):1129-1136, 2001) or the CD14 gene (Furusako et al., Acta Med Okayama. (2):105-115, 2001).

New therapies are needed for septic shock, which carries a high mortality and morbidity. Targeting a component of the LPS receptor complex is an attractive approach since LPS signaling drives a cascade of cytokines that cause fever, vascular permeability and toxic shock.

LPS has also been shown to play a critical role in graft-versus-host disease (Cooke et al. J Clin Invest. 107:1581-9, 2001), and its antagonism with antisense therapy may therefore be beneficial after bone marrow transplantation.

US patent application 2003/0232352 discloses a method of assessing the susceptibility of an individual to atherosclerosis and to a method of treating an individual identified as being at increased risk.

Antisense and Sense Nucleic Acids to Engineer Interference

Antisense technology has been one of the most commonly described approaches in protocols to achieve gene-specific interference. Briefly, A nucleotide sequence is placed under the control of a promoter in a "reverse orientation" such that transcription yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. For antisense strategies, stoichiometric amounts of single-stranded nucleic acid complementary to the messenger RNA for the gene of interest are introduced into the cell.

Antisense has recently become accepted as therapeutic moieties in the treatment of disease states. Representative United States patents that disclose the preparation and methods of such antisense nucleic acids include, but are not limited to U.S. Pat. Nos. 5,004,810; 5,087,617; 5,098,890; 5,135,917; 5,166,195; and 5,194,428.

RNA interference (RNAi) in Gene Silencing

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire et al., Nature 391:806-811, 1998). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing. The process of post-transcriptional gene silencing is thought to be an evolutionarily conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla. Such protection from foreign gene expression may have evolved in response to the presence of double-stranded RNAs (dsRNA) derived from viral infection or the random integration of transposon elements into a host genome, via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response through a mechanism that has yet to be fully characterized. The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III like enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs.

A number of publications disclose methods and oligonucleotide sequences useful as siRNA, see for example WO 00/01846, 99/53050, WO 02/055692, WO02/055693, EP 1144623, WO01/92513 and US Patent No. 6,506,559. International PCT Publications Nos. WO 99/49029 and WO01/70949, and AU 4037501 describe certain vectors expressing siRNA molecules.

Nowhere in the prior art it is taught or suggested that antisense oligonucleotides or double stranded RNA capable of inhibiting the expression of toll-like receptors in general or Tlr4 in particular, may be used for prevention or treatment of septic shock, inflammatory disorders or autoimmune diseases.

SUMMARY OF THE INVENTION

The present invention discloses compositions and methods for inhibiting the expression of the Toll-like receptor-4, particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding Tlr4, ultimately modulating the amount of

Tlr4 produced. This is accomplished by providing oligonucleotides which specifically interact with nucleic acids encoding Tlr4, thereby inhibiting its expression.

The present invention is based on part on the unexpected discovery that oligonucleotides sequences which inhibit the expression of Tlr4, the innate immune
5 receptor for Hsp60 and LPS, are able to modulate the inflammatory process in septic shock as well as in autoimmune conditions where Tlr4 may mediate or contribute to the pathogenesis.

It is an object of the present invention to provide oligonucleotide sequences capable of inhibiting the expression of *tlr4* gene. These oligonucleotide sequences are
10 capable of inhibiting the action of any ligand that acts via the Tlr4 receptor complex.

It is yet another object of the invention to provide modified antisense oligonucleotides capable of sequence specific inhibition of Tlr4 expression.

It is an object of the invention to provide oligonucleotide sequences capable of targeting nucleic acids encoding Tlr4. A preferred target is a gene encoding Tlr4. Another
15 preferred target is an mRNA which encodes Tlr4. The targeting process also includes determination of a site or sites within the nucleic acid sequence for the antisense or dsRNA interaction to occur such that modulation of gene expression will result.

It is a further object of the present invention to provide antisense oligonucleotides or dsRNA molecules capable of downregulating Tlr4. These molecules can cause transient
20 immunosuppression by decreasing the cytokine response during sepsis without the side effects seen with indiscriminate immunosuppressive medications.

A further object of the present invention is to provide pharmaceutical compositions comprising oligonucleotide sequences which are capable of binding to the Tlr4 gene and inhibiting its expression.

25 In one embodiment, preferred molecules according to the present invention are oligonucleotide antisense molecules derived from or complementary to the Tlr4 mRNA. Preferred antisense oligonucleotide sequences have a length of about 5 to about 50 nucleotides, more preferred molecules are 8-30 nucleotides long, and most preferred oligonucleotides are 15-25 long. Specific preferred molecules are DNA sequences, though
30 it is explicitly intended that the scope of the present invention encompasses RNA and PNA (peptide nucleic acid) molecules, as well as oligonucleotides comprising non-natural nucleotide analogs or internucleotidyl linkages.

According to other embodiments preferred molecules are dsRNA oligonucleotides corresponding to the Tlr4 mRNA. Preferred dsRNA oligonucleotide sequences have a length of about 5 to about 40, more preferred oligonucleotide sequences have a length of about 15 to about 25.

5 According to some embodiments the dsRNA corresponds to a fragment of the sequence of the *tlr4* gene. The dsRNA according to the present invention is any dsRNA which inhibits Tlr4 expression via RNA interference (RNAi). In one embodiment, the dsRNAs are synthetic molecules. In another embodiment, an expression vector comprising the dsRNAs is used.

10 Preferred antisense or dsRNA sequences are derived from the human *tlr4* gene sequencedesignated SEQ ID NO:20 [Genebank accession no. AH009665].

Most preferably antisense or dsRNA sequences are derived from a human mRNA transcript variant, one of which is presented in Figure 1 designated SEQ ID NO:1 for the human Tlr4 mRNA transcript 1 [Genebank accession no. NM_138554].

15 As exemplified hereinbelow antisense DNA or dsRNA sequences may be derived from mouse Tlr4 mRNA, the sequence of which is presented in Figure 2 designated as SEQ ID NO:2.

In certain embodiments, the oligonucleotide antisense molecules derived from mouse mRNA are selected from SEQ ID NOs:3-6:

20 5'-TAGGACCGTAAAAGACGTTG-3' designated ODN47, corresponding to bp 24-4 of the mouse Tlr4 mRNA denoted herein as SEQ ID NO:3;

5'-GTGTACAGTAAACAAGTTGT-3' designated ODN48, corresponding to bp 1840-1821 of the mouse Tlr4 mRNA denoted herein as SEQ ID NO:4;

25 5'-GTTACCGATGTGGTCCTTAT-3' designated ODN49, corresponding to bp 2170-2151 of the mouse Tlr4 mRNA denoted herein as SEQ ID NO:5;

5'-AGTTATGAGAAGATTTCGTT-3' designated ODN50, corresponding to bp 2870-2851 of the mouse Tlr4 mRNA denoted herein as SEQ ID NO:6.

In certain embodiments, the dsRNA oligonucleotide molecules derived from mouse mRNA are selected from SEQ ID NOs:7-10:

30 5'-AAGCTTGAATCCCTGCATAGA-3', corresponding to bp 90-110 of the mouse Tlr4 mRNA denoted herein as SEQ ID NO:7

5'-AAACAATTGAAGACAAGGCAT-3', corresponding to bp 290-310 of the mouse Tlr4 mRNA denoted herein as SEQ ID NO:8;

5'-AATTGGCCTCTCTAGAAAGCT-3', corresponding to bp 428-448 of the mouse Tlr4 mRNA denoted herein as SEQ ID NO:9;

- 5 5'- AACCTAGTACATGTGGATCTT-3' corresponding to bp 544-564 of the mouse Tlr4 mRNA denoted herein as SEQ ID NO:10;

In currently more preferred embodiments, the dsRNA oligonucleotide molecules are derived from human mRNA are selected from SEQ ID NOs:11-19:

- 10 5'- GTGGCTGTGGAGACAAATCTA-3', corresponding to bp 681-701 of the human Tlr4 mRNA denoted herein as SEQ ID NO:11;

5'- GAATTCCGATTAGCATACTTA-3', corresponding to bp 1146-1166 of the human Tlr4 mRNA denoted herein as SEQ ID NO:12;

5'- GATTAGCATACTTAGACTACT-3', corresponding to bp 1153-1173 of the human Tlr4 mRNA denoted herein as SEQ ID NO:13;

- 15 5'- GGATGGCAACATTTAGAATTA-3', corresponding to bp 1278-1298 of the human Tlr4 mRNA denoted herein as SEQ ID NO:14;

5'- GCTTGTCCAGTCTCGAAGTCT-3', corresponding to bp 1693-1713 of the human Tlr4 mRNA denoted herein as SEQ ID NO:15;

- 20 5'- GGTAAGGAATGAGCTAGTAAA-3', corresponding to bp 2348-2368 of the human Tlr4 mRNA denoted herein as SEQ ID NO:16;

5'- GAGGGAATAAATGCTAGACTA-3', corresponding to bp 2976-2996 of the human Tlr4 mRNA denoted herein as SEQ ID NO:17;

5'- GGTCATTCTCGAGCATGTTCT-3', corresponding to bp 3380-3400 of the human Tlr4 mRNA denoted herein as SEQ ID NO:18;

5'-GTCATTCTCGAGCATGTTCTA-3', corresponding to bp 3381-3401 of the human Tlr4 mRNA denoted herein as SEQ ID NO:19;

The genomic sequence of human *tlr4*, denoted herein as SEQ ID NO:20, is presented in Figure 3.

5 In currently preferred embodiments, the oligonucleotide antisense molecules derived from human mRNA are selected from SEQ ID NOs:21-24:

5'-GCTTCAGTTCCTCACCTAT-3', corresponding to bp 85-67 of the human Tlr4 mRNA denoted herein as SEQ ID NO:21;

10 5'-TTGGGTTTGGTGTTCCTGC-3', corresponding to bp 448-430 of the human Tlr4 mRNA denoted herein as SEQ ID NO:22;

5'-TCCCACTTCCTTCCTGCCTCT-3', corresponding to bp 488-461 of the human Tlr4 mRNA denoted herein as SEQ ID NO:23;

5'-CCTCATTTCTCCCTTCCTCC-3', corresponding to bp 5877-5858 of the human Tlr4 mRNA denoted herein as SEQ ID NO:24;

15 According to yet another aspect the present invention provides a pharmaceutical composition comprising as an active ingredient an oligonucleotide molecule comprising a contiguous nucleotide sequence from a group consisting of a fragment of the genomic sequence of *tlr4* or an mRNA transcribed therefrom.

20 According to some embodiments the pharmaceutical compositions comprise any pharmaceutically acceptable excipients, diluents or carriers. According to some embodiments the oligonucleotide molecules are inserted into constructs. According to certain embodiment the oligonucleotide molecules are incorporated into an expression.

25 Further aspects of the present invention provides methods for preventing or treating a disease or disorder, comprising administering to a subject in need thereof a pharmaceutical composition comprising a therapeutically effective amount of an oligonucleotide for inhibiting Toll-like receptor 4 gene expression wherein the oligonucleotide comprises a contiguous nucleotide sequence selected from the group consisting of a fragment of the genomic sequence of *tlr4* and an mRNA transcribed therefrom.

The antisense oligonucleotides and dsRNA molecules disclosed in the present invention can prolong survival in septic shock and may therefore be beneficial as adjunctive therapy in its treatment.

5 According to the present invention LPS antagonism with antisense or dsRNA molecules specific to Tlr4 may be beneficial in the treatment of gram negative bacterial sepsis even without the signs and symptoms of shock. The oligonucleotide sequences according to the present invention may be also used after bone marrow transplantation, due to the known involvement of LPS in graft versus host disease.

10 The pro-inflammatory signaling of human hsp60 was found to be dependent on a functional Tlr4. This finding suggests the existence of endogenous ligands of the Tlr4 complex, and a role of Toll-like receptors in innate immune discrimination of normal versus stressed or damaged tissue cells. It is therefore another aspect of the invention to provide molecules which, by blocking Tlr4, the innate immune receptor for Hsp60, will have a beneficial effect in inflammatory diseases shown to involve Hsp60. These diseases
15 include but are not limited to: arthritis, type 1 diabetes, multiple sclerosis, inflammatory bowel disease, and atherosclerosis.

Accordingly, the present invention relates to a method for the treatment or prevention of bacterial septic shock by administering to a subject a pharmaceutical composition comprising as an active ingredient oligonucleotide molecules according to the
20 present invention.

According to one embodiment the subject of the present invention is of mammalian origin. According to some embodiments the subject of the present invention is of human origin.

25 Use of a molecule according to the present invention for the preparation of pharmaceutical compositions for treatment or prevention of sepsis, and inflammatory or autoimmune diseases, represents another aspect of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Homo sapiens toll-like receptor 4 (Tlr4) mRNA transcript variant 1 designated SEQ ID NO:1.

Figure 2 Mus musculus toll-like receptor 4 (Tlr4) mRNA, designated SEQ ID NO:2.

5 **Figure 3** Homo sapiens toll-like receptor 4 (*tlr4*) genomic sequence designated SEQ ID NO:20.

Figure 4 A graph showing the percent survival of mice at 48 hours after intravenous challenge with 1 mg LPS. The control, ODN47, ODN48 and ODN49 groups each had 5 mice/group. The ODN50 group had 4 mice.

10 **Figure 5** A graph showing serum TNF α levels at 90 minutes after LPS challenge.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligonucleotide molecules for use in modulating
15 the function of nucleic acids encoding Tlr4, ultimately modulating the amount of Tlr4 produced. This is accomplished by providing oligonucleotides which specifically hybridize with nucleic acids encoding Tlr4, preferably hybridizing with mRNA transcripts encoding Tlr4, most preferably oligonucleotides specifically hybridizing with human Tlr4.

For inhibition of septic shock such as that elicited by LPS, Tlr4 may be a more
20 appropriate target than TNF α since there are likely to be additional cytokines that contribute to the complications of sepsis. Suppressing Tlr4 is likely to be safer than CD14 suppression since CD14 participates as a co-receptor of other microbial products, whereas Tlr4 is specific for the LPS of gram negative bacteria.

Targeting an upstream event in the inflammatory cascade (the LPS receptor) may
25 be more effective than inhibiting the high level of a particular circulating cytokine, since even partial suppression of the LPS response pathway at the genetic level can significantly affect multiple downstream events. Mice lacking functional Tlr4 are resistant to lethal doses of LPS. According to the present invention antisense oligonucleotides or dsRNA specific for Tlr4 are shown to provide protection from LPS induced septic shock in a
30 murine model and therefore may be used for treatment of septic shock in mammals.

The pro-inflammatory signaling of human hsp60 was found to be dependent on a functional Tlr4. This finding suggests the existence of endogenous ligands of the Tlr4 complex, and a role of Toll-like receptors in innate immune discrimination of normal versus stressed or damaged tissue cells. The present invention provides molecules which, by blocking Tlr4, the innate immune receptor for Hsp60, will have a beneficial effect in inflammatory diseases shown to involve Hsp60. These diseases include but are not limited to: arthritis, type 1 diabetes, multiple sclerosis, inflammatory bowel disease, and atherosclerosis. Similarly, other endogenous ligands of Toll-like receptors have been described and reducing the signaling by these molecules may be beneficial in particular clinical situations (Beg, AA., Trends Immunol. 23:509-512, 2002).

Transient immunosuppression caused by downregulating Tlr4 with antisense oligonucleotides or dsRNA can decrease the cytokine response during sepsis without the side effects seen with indiscriminate immunosuppressive medications.

According to the present invention, septic shock is blocked by downregulating the LPS receptor protein Tlr4 using inhibitory oligonucleotide sequences such as an antisense oligonucleotide or a dsRNA. Antisense oligonucleotides can suppress the expression of particular genes in a sequence-dependent manner both *in vitro* and *in vivo*. Hybridization of antisense oligonucleotides with mRNA interferes with one or more of the normal functions of the mRNA. The functions of mRNA to be interfered include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in by the RNA. Binding of specific protein(s) to the RNA may also be interfered with by antisense oligonucleotide hybridization to the RNA.

Use of antisense oligonucleotides is well known in the art. For example US 6,228,642 describes methodology and terminology related to antisense technology. This patent is incorporated here in its entirety by reference. The following text includes non-limitative examples for such methods and terminology.

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire et al., Nature 391:806-811, 1998). The corresponding process in plants is commonly referred to as post-

transcriptional gene silencing or RNA silencing. The process of post-transcriptional gene silencing is thought to be an evolutionary conserved cellular defense mechanism for controlling the expression of alien genes in filamentous fungi, plants, and animals (Hannon GJ. Nature. 2002 418:244-251). It is caused by sequence-specific mRNA degradation, and
5 is mediated by dsRNA homologous in sequence to the target RNA. dsRNA is often a byproduct of viral replication or is formed by aberrant transcription from genetic elements after random integration in the host genome. dsRNA is processed to duplexes of 21-nt small interfering RNAs (siRNAs), which guide sequence-specific degradation of the homologous mRNA. The RNAi pathway can be used by the organism to inhibit viral
10 infections, transposon jumping and to regulate the expression of endogenous genes.

As used herein, the terms RNA, RNA molecules and RNA fragments are used interchangeably to refer to RNA that mediates RNA interference. These terms include double-stranded RNA, single-stranded RNA, isolated RNA (partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA), as well as altered
15 RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides.

The relationship between an antisense compound such as an oligonucleotide and its complementary nucleic acid target, to which it hybridizes, is commonly referred to as "antisense". "Targeting" an oligonucleotide to a chosen nucleic acid target, in the context of
20 this invention, is a multistep process. The process usually begins with identifying a nucleic acid sequence whose function is to be modulated. This may be, as examples, a cellular gene (or a gene transcript) whose expression is associated with a particular disease state, or a foreign nucleic acid from an infectious agent. In the present invention, the targets are nucleic acids encoding Tlr4; in other words, a gene encoding Tlr4, or mRNA expressed
25 from the Tlr4 gene. mRNA which encodes Tlr4 is presently the preferred target. The targeting process also includes determination of a site or sites within the nucleic acid sequence for the antisense interaction to occur such that modulation of gene expression will result.

In accordance with this invention, persons of ordinary skill in the art will
30 understand that messenger RNA includes not only the information to encode a protein using the three letter genetic code, but also associated ribonucleotides which form a region

known to such persons as the 5'-untranslated region, the 3'-untranslated region, the 5' cap region and intron/exon junction ribonucleotides. Thus, oligonucleotides may be formulated in accordance with this invention which are targeted wholly or in part to these associated ribonucleotides as well as to the informational ribonucleotides. The oligonucleotide may therefore be specifically hybridizable with a transcription initiation site region, a translation initiation codon region, a 5' cap region, an intron/exon junction, coding sequences, a translation termination codon region or sequences in the 5'- or 3'-untranslated region.

Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon." A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding Tlr4, regardless of the sequence(s) of such codons. It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region," "AUG region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. This region is a preferred target region.

Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. This region is a preferred target region. The open reading frame (ORF)

or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other preferred target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'--5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a pre-mRNA transcript to yield one or more mature mRNAs. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., exon-exon or intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. Targeting particular exons in alternatively spliced mRNAs may also be preferred. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once the target site or sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired modulation.

"Hybridization", in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between

them. Adenine and thymine are examples of complementary bases which form two hydrogen bonds between them.

"Specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs
5 between the DNA or RNA target and the oligonucleotide.

It is to be understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient
10 degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment or, in the case of *in vitro* assays, under conditions in which the assays are conducted.

As 21-nucleotide siRNA duplexes were found to be highly selective and
15 sequence-specific inhibitors of endogenous genes, the present invention encompasses siRNA sequences comprising at least 15 nucleotides, more preferably at least 20 nucleotides. The present invention, therefore, encompasses double stranded RNA (dsRNA), and more particularly small interfering RNA (siRNA), which are known to mediate sequence specific mRNA degradation.

20 Identification of desirable dsRNA sequences to inhibit the human *tlr 4* or murine gene is done by using various algorithm programs. BLAST search and FastA sequence alignment computer programs are used in combination and sequences are chosen that do not display a high degree of homology to other genes in the genome. This strategy avoids non-desired gene suppression.

25 It should be appreciated that the present invention provides siRNA sequences that are highly specific to the *tlr4* gene and will not cross-react with other murine or human genes. These sequences typically share 100% homology to the target gene or mRNA and show low identity of at least four mismatched nucleotides corresponding to other genes in the murine and human genome. Nevertheless it will be appreciated that 100% sequence
30 identity between the RNA and the target gene is not required to practice the present

invention. Thus, the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

5 The overall effect of interference with mRNA function is modulation of expression of Tlr4. In the context of this invention "modulation" means either inhibition or stimulation; i.e., either a decrease or increase in expression. This modulation can be measured in ways which are routine in the art, for example by Northern blot assay of mRNA expression, or reverse transcriptase PCR, as taught in the examples of the instant application or by Western blot or ELISA assay of protein expression, or by an
10 immunoprecipitation assay of protein expression. Effects of antisense oligonucleotides of the present invention on Tlr4 expression can also be determined as taught in the examples of the instant application. Inhibition is presently a preferred form of modulation.

Gene-specific inhibition of gene expression by double-stranded ribonucleic acid (dsRNA) is disclosed for example in US Patent No. 6,506,559. This patent is incorporated
15 herein in its entirety by reference. The following text includes non-limitative examples for such methods and terminology.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery
20 of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

25 Other alternative nucleic acid molecules that are capable of attenuating gene expression include but are not limited to triple-stranded nucleic acids, enzymatic nucleic acid molecules and aptamers.

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through
30 the modification of polynucleotide sequences that encode these RNAs, it is possible to engineer molecules that recognize specific polynucleotide sequences associated with

production of a mutated proto-oncogene or tumor suppressor gene in an RNA molecule and cleave it (Cech, J. Amer. Med. Assn., 260:3030, 1988). A major advantage of this approach is that, because ribozymes are sequence-specific, only target mRNAs with particular mutant sequences are inactivated.

5 RNA containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press,
10 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred.
15 Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C. or 70°C. hybridization for 12-16 hours; followed by washing). The length of the identical nucleotide sequences may be at least 25, 50, 100, 200, 300 or 400 bases.

20 As disclosed herein, 100% sequence identity between the inhibitory oligonucleotide and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

RNA may be synthesized either in vivo or in vitro. Endogenous RNA polymerase
25 of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For transcription from a transgene in vivo or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an
30 environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into

a polypeptide by a cell's translational apparatus. RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art (see WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by in vitro enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing the RNA. Methods for oral introduction include direct mixing of the RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express the RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids, for example, injection directly into the cell or extracellular injection into the organism, may also be used. Vascular or extravascular circulation, the blood or lymph system, the phloem, the roots, and the cerebrospinal fluid are sites where the RNA may be introduced.

Any cell that undergoes RNAi can be employed in methods of the invention. The cell may be a eukaryotic cell. Typically, the cell is of animal origin and can be a stem cell or somatic cells. Suitable animal cells can be of, for example, mammalian and avian origin. Examples of mammalian cells include human, bovine, ovine, porcine, murine, rabbit cells. The cell may be an embryonic cell, bone marrow stem cell or other progenitor cell. Where the cell is a somatic cell, the cell can be, for example, an epithelial cell, fibroblast, smooth muscle cell, blood cell (including a hematopoietic cell, red blood cell, T-cell, B-cell, etc.), tumor cell, cardiac muscle cell, macrophage, dendritic cell, neuronal cell (e.g., a glial cell

or astrocyte); or pathogen-infected cell (e.g., those infected by bacteria, viruses, virusoids, parasites, or prions).

An expression vector comprising an siRNA may be used to introduce the siRNA into a cell. These expression vectors can be used either *in vitro* or *in vivo* and cells can be stably transfected ex vivo prior to transfer to a host. siRNA translated from a vector forms a stem and loop structure that is recognized inside the cell by a protein complex. The elements required in the vector include first and foremost an appropriate promoter for translation of the DNA to RNA in the selected system. Most vectors currently employ the RNA polymerase III promoters U6 or H1 since RNA polymerase III initiates synthesis at a defined distance from these promoters and terminates when a string of 4-5 uridines is encountered. RNAi activation *in vivo* can be produced by using a vector which expresses an siRNA under control of inducible or tissue-specific promoters thereby allowing directed and controlled knockout of genes in tissues of interest and/or at a controlled time (Lois C, et al., Science. 295:868-872, 2002). Active short interfering RNAs can also be produced under the control of the tRNA (Val) promoter or polymerase II (pol II) promoter.

"RNAi expression vector" (also referred to herein as a "dsRNA-encoding plasmid") refers to a replicable nucleic acid constructs used to express (transcribe) RNA which produces siRNA moieties in the cell in which the construct is expressed. Such vectors include a transcriptional unit comprising an assembly of (1) genetic element(s) having a regulatory role in gene expression, for example, promoters, operators, or enhancers, operatively linked to (2) a "coding" sequence which is transcribed to produce a double-stranded RNA (two RNA moieties that anneal in the cell to form an siRNA, or a single hairpin RNA which can be processed to an siRNA), and (3) appropriate transcription initiation and termination sequences. The choice of promoter and other regulatory elements generally varies according to the intended host cell. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. In the expression vectors, regulatory elements controlling transcription can be generally derived from mammalian, microbial, viral or insect genes. The ability to replicate in a host, usually conferred by an origin of replication,

and a selection gene to facilitate recognition of transformants may additionally be incorporated.

The term "tissue specific" means a promoter which is not restricted in activity to a single tissue type but which nevertheless shows selectivity in that they may be active in one group of tissues and less active or silent in another group.

The expression level of an oligonucleotide sequence of interest under the control of a particular promoter may be modulated by manipulating the promoter region. For example, different domains within a promoter region may possess different gene regulatory activities. The roles of these different regions are typically assessed using vector constructs having different variants of the promoter with specific regions deleted (that is, deletion analysis). This approach may be used to identify, for example, the smallest region capable of conferring tissue specificity. In most instances, these promoters may be isolated as convenient restriction digestion fragments suitable for cloning in a selected vector. Alternatively, promoter fragments may be isolated using the polymerase chain reaction. Cloning of the amplified fragments may be facilitated by incorporating restriction sites at the 5' end of the primers.

For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucuronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

Vectors that may include the above described components exist publicly and commercially. Examples for *in vitro* RNAi vector-based system include pSuper (Brummelkamp TR et al., Science 296:550-553, 2002) and pSilencer (Sui G. et al., Proc Natl Acad Sci 99:5515-5520, 2002) which generate long-term loss-of-function phenotypes in mammalian cells.

According to certain preferred embodiments of the present invention, the siRNA directed against the TLR4 target sequences is inserted into a Lentiviral vector.

Lentivirus vectors are part of a larger group of retroviral vectors. A detailed list of lentiviruses may be found in Coffin et al ("Retroviruses" 1997 Cold Spring Harbour Laboratory Press Eds: J M Coffin, S M Hughes, H E Varmus pp 758-763). In brief, lentiviruses can be divided into primate and non-primate groups. Examples of primate lentiviruses include but are not limited to: the human immunodeficiency virus (HIV), the causative agent of human auto-immunodeficiency syndrome (AIDS), and the simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV). Lentiviruses have key advantages over other gene delivery systems.

A lentiviral or lentivirus vector, as used herein, is a vector which comprises at least one component part derivable from a lentivirus. Preferably, that component part is involved in the biological mechanisms by which the vector infects cells, expresses genes or is replicated. The term "derivable" is used in its normal sense as meaning the sequence need not necessarily be obtained from a retrovirus but instead could be derived therefrom. By way of example, the sequence may be prepared synthetically or by use of recombinant DNA techniques.

Lentiviruses infect multiple cell types and efficiently infect non-dividing cells. Genes expressed from lentiviruses are not silenced during development therefore transgenic animals can be generated by lentiviral infection of embryonic stem cells or embryos (Lois C et al., Science. 295:868-72, 2002). In addition, lentiviral vectors enable very stable long-term expression of the gene of interest.

Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct. Other methods known in the art for introducing nucleic acids to

cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or otherwise increase inhibition of the target gene.

The oligonucleotides of this invention can be used in diagnostics, therapeutics, prophylaxis, as research reagents and in kits. Since the oligonucleotides of this invention hybridize to nucleic acids encoding Tlr4, sandwich, colorimetric and other assays can easily be constructed to exploit this fact. Provision of means for detecting hybridization of oligonucleotides with the Tlr4 gene or mRNA can routinely be accomplished. Such provision may include enzyme conjugation, radiolabelling or any other suitable detection systems. Kits for detecting the presence or absence of Tlr4 may also be prepared.

The present invention is also suitable for diagnosing abnormal inflammatory states in tissue or other samples from patients suspected of having an inflammatory disease associated with hsp60. The ability of the oligonucleotides of the present invention to inhibit inflammatory processes may be employed to diagnose such states. A number of assays may be formulated employing the present invention, which assays will commonly comprise contacting a tissue sample with an oligonucleotide of the invention under conditions selected to permit detection and, usually, quantitation of such inhibition. In the context of this invention, to "contact" tissues or cells with an oligonucleotide or oligonucleotides means to add the oligonucleotide(s), usually in a liquid carrier, to a cell suspension or tissue sample, either in vitro or ex vivo, or to administer the oligonucleotide(s) to cells or tissues within an animal.

The oligonucleotides of this invention may also be used for research purposes. Thus, the specific hybridization exhibited by the oligonucleotides may be used for assays, purifications, cellular product preparations and in other methodologies which may be appreciated by persons of ordinary skill in the art.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which

function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases. In addition, the oligonucleotide sequences may contain one or more nucleotide substitution
5 which does not render the efficiency of hybridization to the target sequence.

The antisense compounds in accordance with this invention preferably comprise from about 5 to about 50 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 nucleobases (i.e. from about 8 to about 30 linked nucleosides). Most preferably are antisense oligonucleotides comprising from about 15 to
10 about 25 nucleobases. As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate
15 group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are
20 commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones
25 include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example,
30 phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene

phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity
5 wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302;
10 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl
15 internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene
20 containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315;
25 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside
30 linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound.

One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos.: 5,539,082; 5,714,331; and 5,719,262. Further teaching of PNA compounds can be found in Nielsen et al. (Science, 1991, 254, 1497-1500).

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-\text{CH}_2\text{-NH-O-CH}_2-$, $-\text{CH}_2\text{-N(CH}_3\text{)-O-CH}_2-$ [known as a methylene (methylimino) or MMI backbone], $-\text{CH}_2\text{-O-N(CH}_3\text{)-CH}_2-$, $-\text{CH}_2\text{-N(CH}_3\text{)-N(CH}_3\text{)-CH}_2-$ and $-\text{O-N(CH}_3\text{)-CH}_2\text{-CH}_2-$ [wherein the native phosphodiester backbone is represented as $-\text{O-P-O-CH}_2-$] of the above referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above referenced U.S. Pat. No. 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl, O-alkyl-O-alkyl, O-, S-, or N-alkenyl, or O-, S- or N-alkynyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C1 to C10 alkyl or C2 to C10 alkenyl and alkynyl. Particularly preferred are $\text{O}[(\text{CH}_2)_n \text{O}]_m \text{CH}_3$, $\text{O}(\text{CH}_2)_n \text{OCH}_3$, $\text{O}(\text{CH}_2)_2 \text{ON}(\text{CH}_3)_2$, $\text{O}(\text{CH}_2)_n \text{NH}_2$, $\text{O}(\text{CH}_2)_n \text{CH}_3$, $\text{O}(\text{CH}_2)_n \text{ONH}_2$, and $\text{O}(\text{CH}_2)_n \text{ON}[(\text{CH}_2)_n \text{CH}_3]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C1 to C10 lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂ CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification

includes 2'-methoxyethoxy (2'-O-CH₂ CH₂ OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta* 1995, 78, 486-504) i.e., an alkoxyalkoxy group. Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-aminopropoxy (2'-OCH₂ CH₂ CH₂ NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C or m5c), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in the *Concise Encyclopedia Of Polymer Science And Engineering* 1990, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, those disclosed by Englisch et al. (*Angewandte Chemie, International Edition* 1991, 30, 613-722), and those disclosed by Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., eds., *Antisense Research and Applications* 1993, CRC Press, Boca Raton, pages 289-302.

Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-Methylcytosine
5 substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2.degree. C. (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., eds., *Antisense Research and Applications* 1993, CRC Press, Boca Raton, pages 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

10 Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. Nos. 3,687,808, as well as U.S. Pat. No. 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617;
15 and 5,681,941.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al.,
20 Proc. Natl. Acad. Sci. USA 1989, 86, 6553-6556), cholic acid (Manoharan et al., Bioorg. Med. Chem. Lett. 1994, 4, 1053-1059), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., Ann. N.Y. Acad. Sci. 1992, 660, 306-309; Manoharan et al., Bioorg. Med. Chem. Lett. 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., Nucl. Acids Res. 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al.,
25 EMBO J. 1991, 10, 1111-1118; Kabanov et al., FEBS Lett. 1990, 259, 327-330; Svinarchuk et al., Biochimie 1993, 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., Tetrahedron Lett. 1995, 36, 3651-3654; Shea et al., Nucl. Acids Res. 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al.,
30 Nucleosides & Nucleotides 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett. 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., Biochim. Biophys. Acta 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-

oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther. 1996, 277, 923-937). Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941.

The present invention also includes oligonucleotides which are chimeric oligonucleotides. "Chimeric" oligonucleotides or "chimeras," in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. This RNase H-mediated cleavage of the RNA target is distinct from the use of ribozymes to cleave nucleic acids. Ribozymes are not comprehended by the present invention.

Examples of chimeric oligonucleotides include but are not limited to "gapmers," in which three distinct regions are present, normally with a central region flanked by two regions which are chemically equivalent to each other but distinct from the gap. A preferred example of a gapmer is an oligonucleotide in which a central portion (the "gap") of the oligonucleotide serves as a substrate for RNase H and is preferably composed of 2'-deoxynucleotides, while the flanking portions (the 5' and 3' "wings") are modified to have

greater affinity for the target RNA molecule but are unable to support nuclease activity (e.g., fluoro- or 2'-O-methoxyethyl-substituted). Chimeric oligonucleotides are not limited to those with modifications on the sugar, but may also include oligonucleosides or oligonucleotides with modified backbones, e.g., with regions of phosphorothioate and phosphodiester backbone linkages. Other chimeras include "wingmers," also known in the art as "hemimers," that is, oligonucleotides with two distinct regions. In a preferred example of a wingmer, the 5' portion of the oligonucleotide serves as a substrate for RNase H and is preferably composed of 2'-deoxynucleotides, whereas the 3' portion is modified in such a fashion so as to have greater affinity for the target RNA molecule but is unable to support nuclease activity (e.g., 2'-fluoro- or 2'-O-methoxyethyl-substituted), or vice-versa. In one embodiment, the oligonucleotides of the present invention contain a 2'-O-methoxyethyl (2'-O-CH₂ CH₂ OCH₃)₃ modification on the sugar moiety of at least one nucleotide. This modification has been shown to increase both affinity of the oligonucleotide for its target and nuclease resistance of the oligonucleotide. According to the invention, one, a plurality, or all of the nucleotide subunits of the oligonucleotides of the invention may bear a 2'-O-methoxyethyl (-O-CH₂ CH₂ OCH₃) modification. Oligonucleotides comprising a plurality of nucleotide subunits having a 2'-O-methoxyethyl modification can have such a modification on any of the nucleotide subunits within the oligonucleotide, and may be chimeric oligonucleotides. Aside from or in addition to 2'-O-methoxyethyl modifications, oligonucleotides containing other modifications which enhance antisense efficacy, potency or target affinity are also preferred. Chimeric oligonucleotides comprising one or more such modifications are presently preferred.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routine experimenter. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and 2'-alkoxy or 2'-alkoxyalkoxy derivatives, including 2'-O-methoxyethyl oligonucleotides (Martin, P., *Helv. Chim. Acta* 1995, 78, 486-504). It is also well known to use similar techniques and commercially available modified amidites and controlled-pore glass (CPG) products such as biotin, fluorescein, acridine or psoralen-modified amidites and/or CPG

(available from Glen Research, Sterling, Va.) to synthesize fluorescently labeled, biotinylated or other conjugated oligonucleotides.

The antisense compounds of the present invention include bioequivalent compounds, including pharmaceutically acceptable salts and prodrugs. This is intended to encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to pharmaceutically acceptable salts of the nucleic acids of the invention and prodrugs of such nucleic acids. "Pharmaceutically acceptable salts" are physiologically and pharmaceutically acceptable salts of the nucleic acids of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto (see, for example, Berge et al., "Pharmaceutical Salts," J. of Pharma Sci. 1977, 66, 1-19).

For oligonucleotides, examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The oligonucleotides of the invention may additionally or alternatively be prepared to be delivered in a "prodrug" form. The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510.

For therapeutic or prophylactic treatment, oligonucleotides are administered in accordance with this invention. Oligonucleotide compounds of the invention may be formulated in a pharmaceutical composition, which may include pharmaceutically acceptable carriers, thickeners, diluents, buffers, preservatives, surface active agents, neutral or cationic lipids, lipid complexes, liposomes, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients and the like in addition to the oligonucleotide. Such compositions and formulations are comprehended by the present invention.

Pharmaceutical compositions comprising the oligonucleotides of the present invention may include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, i.e., fatty acids, bile salts, chelating agents, surfactants and non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems* 1991, 8, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems* 1990, 7, 1-33). One or more penetration enhancers from one or more of these broad categories may be included. Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, recinleate, monoolein (a.k.a. 1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, mono- and di-glycerides and physiologically acceptable salts thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems* 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems* 1990, 7, 1; El-Hariri et al., *J. Pharm. Pharmacol.* 1992 44, 651-654).

The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 In: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, N.Y., 1996, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives.

Complex formulations comprising one or more penetration enhancers may be used. For example, bile salts may be used in combination with fatty acids to make complex formulations.

Chelating agents include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines)(Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems 1990, 7, 1-33; Buur et al., J. Control Rel. 1990, 14, 43-51). Chelating agents have the added advantage of also serving as DNase inhibitors.

Surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems 1991, page 92); and perfluorochemical emulsions, such as FC-43 (Takahashi et al., J. Pharm. Pharmacol. 1988, 40, 252-257).

Non-surfactants include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., J. Pharm. Pharmacol. 1987, 39, 621-626).

As used herein, "carrier compound" refers to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized as a nucleic acid by in vivo processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. In contrast to a carrier compound, a "pharmaceutically acceptable carrier" (excipient) is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The pharmaceutically acceptable carrier may be liquid or solid and is selected with the planned manner of administration in mind so as to provide for the

desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition.

Typical pharmaceutically acceptable carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrates (e.g., starch, sodium starch glycolate, etc.); or wetting agents (e.g., sodium lauryl sulphate, etc.). Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are described in U.S. Pat. Nos. 4,704,295; 4,556,552; 4,309,406; and 4,309,404.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional compatible pharmaceutically-active materials such as, e.g., antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the oligonucleotides of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to enhance the in vivo stability of the oligonucleotides and/or to target the oligonucleotides to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal dispersion system is a plurality of liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layers made up of lipids arranged in a bilayer configuration (see, generally, Chonn et al., *Current Op. Biotech.* 1995, 6, 698-708).

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, epidermal, and transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. In some cases it may be more effective to treat a patient with an oligonucleotide of the invention in conjunction with other traditional therapeutic modalities in order to increase the efficacy of a treatment regimen. In the context of the invention, the term "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. For example, a patient may be treated with conventional chemotherapeutic agents such as those used for tumor and cancer treatment. When used with the compounds of the invention, such chemotherapeutic agents may be used individually, sequentially, or in combination with one or more other such chemotherapeutic agents.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary

depending on the relative potency of individual oligonucleotides, and can generally be estimated based on the EC₅₀ found to be effective during *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 µg to 100 mg per kg of body weight, and may be given once or more daily, or at longer intervals. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 µg to 100 mg per kg of body weight, once or more daily, or at longer intervals if appropriate.

Thus, in the context of this invention, by "therapeutically effective amount" is meant the amount of the compound which is required to have a therapeutic effect on the treated individual. This amount, which will be apparent to the skilled artisan, will depend upon the age and weight of the individual, the type of disease to be treated, perhaps even the gender of the individual, and other factors which are routinely taken into consideration when designing a drug treatment. A therapeutic effect is assessed in the individual by measuring the effect of the compound on the disease state.

The invention is exemplified by the following non-limitative examples:

EXAMPLES

Antisense oligonucleotides

Four 20 bp antisense oligonucleotides were designed against different regions of the murine Tlr4 gene. The program M fold was used to identify regions of the Tlr4 mRNA predicted to have limited tertiary structure. The sequences used were:

ODN47 5'-TAGGACCGTAAAAGACGTTG-3' (corresponding to bp 24-4 of the Tlr4 mRNA);

ODN48 5'-GTGTACAGTAAACAAGTTGT-3' (bp 1840-1821);

ODN49 5'-GTTACCGATGTGGTCCTTAT-3' (bp 2170-2151);

ODN50 5'-AGTTATGAGAAGATTTTCGTT-3' (bp 2870-2851).

The oligonucleotides were synthesized with 3 phosphorothioate linkages on each end to enhance stability. Desalting was carried out with NAP-25 columns (Pharmacia).

dsRNA oligonucleotides

Four 21 nucleotides of single strands oligonucleotides are designed against
5 different regions of the murine *tlr4* gene. These sequences are shown as single stranded stretches, however, for siRNA double stranded RNA oligonucleotides, are created by hybridization of a complementary oligonucleotides, leaving a 2 nucleotide overhang on the 3' end.

5'- AAGCTTGAATCCCTGCATAGA-3'

10 5'-AAACAATTGAAGACAAGGCAT-3'

5'- AATTGGCCTCTCTAGAAAGCT-3'

5'- AACCTAGTACATGTGGATCTT-3'

Nine 21 nucleotides of single strands oligonucleotides are designed against different regions of the human *Tlr4* gene.

15 5'- GTGGCTGTGGAGACAAATCTA-3'

5'- GAATTCCGATTAGCATACTTA-3'

5'- GATTAGCATACTTAGACTACT-3'

5'- GGATGGCAACATTTAGAATTA-3'

5'- GCTTGTCCAGTCTCGAAGTCT -3'

20 5'-GGTAAGGAATGAGCTAGTAAA-3'

5'-GAGGGAATAAATGCTAGACTA-3'

5'-GGTCATTCTCGAGCATGTTCT-3'

5'- GTCATTCTCGAGCATGTTCTA-3'

25 Plasmids

siRNA expression vectors: for generating the siRNA expression vectors pSilencer (Sui G et al., Proc Natl Acad Sci 99(8):5515-5520, 2002) or pSuper (Brummelkamp TR et al. Science 296:550-553, 2002) are preferably used.

30 LPS induced shock

Six-week old female E α NOD mice were divided into 5 groups. These mice are bred in a clean facility at the Weizmann Institute. 75 micrograms of each oligonucleotide in

200µl dH₂O was administered intravenously (i.v.) at -42 and -20 hours prior to challenge with LPS. Control animals received 200 ul of dH₂O. The mice were challenged i.v. with 1 mg LPS *E.coli* 0127:B8 (Sigma, Israel). Mice were bled from the retroorbital venous plexus 90 minutes after LPS challenge and the sera were tested for murine TNFα using a sandwich ELISA (Endogen) according to manufacturer instructions. Percent survival of the mice was recorded at 48 hrs (see Figure 4).

Rescuing mice from LPS induced septic shock

All control animals died by 48 hours. In contrast, all four antisense oligonucleotides showed some protective effect as shown in Figure 4, with ODN47 being the most beneficial. All control animals had high serum levels of TNFα 90 minutes after challenge with LPS. In contrast, none of the 5 mice treated with ODN47 or ODN48 had detectable TNFα. Two mice in the ODN49 and two in the ODN50 groups had detectable TNFα as presented in Figure 5. Therefore, antisense oligonucleotides to Tlr4 can downregulate circulating TNFα and prolong survival in a murine model of septic shock.

The foregoing description of the specific embodiments so fully reveals the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention.

WHAT IS CLAIMED IS:

1. An oligonucleotide for inhibiting Toll-like receptor 4 (Tlr4) gene expression, wherein said oligonucleotide comprises a contiguous nucleotide sequence corresponding to a fragment of the genomic sequence of Tlr4 or an mRNA transcribed therefrom.
- 5 2. The oligonucleotide of claim 1 wherein the oligonucleotide sequence is selected from the group consisting of antisense, a double-stranded RNA (dsRNA), triple-stranded nucleic acids, enzymatic nucleic acid molecules and aptamers.
3. The oligonucleotide of claim 2 wherein the oligonucleotide sequence is selected from the group consisting of antisense and double-stranded RNA (dsRNA).
- 10 4. An antisense oligonucleotide for inhibiting Toll-like receptor 4 (*tlr4*) gene expression, wherein said oligonucleotide comprises a contiguous nucleotide sequence selected from the group consisting of a fragment of SEQ ID NO:1, a fragment of SEQ ID NO:2 or a fragment of SEQ ID NO:20.
- 15 5. The antisense oligonucleotide of claim 4 selected from any one of SEQ ID NOs: 3-6, and SEQ ID NOS: 21-24, or an active fragment thereof.
6. The antisense oligonucleotide of claim 4 comprising 5 to 50 nucleotides.
7. The antisense oligonucleotide of claim 6 comprising 8 to 30 nucleotides.
8. The antisense oligonucleotide of claim 7 comprising 15 to 25 nucleotides.
- 20 9. An antisense oligonucleotide comprising 5 to 50 nucleotides, wherein said oligonucleotide comprises a contiguous nucleotide sequence corresponding to a fragment selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO:20 which specifically hybridizes to an mRNA encoding human Tlr4.
- 25 10. A double-stranded RNA (dsRNA) oligonucleotide for inhibiting Toll-like receptor 4 (*tlr4*) gene expression wherein said oligonucleotide comprises a contiguous nucleotide sequence selected from the group consisting of a fragment of SEQ ID NO:1, a fragment of SEQ ID NO:2, and a fragment of SEQ ID NO:20.
11. The dsRNA of claim 10 selected from the group consisting and of SEQ ID NOs:7-19, or an active fragment thereof..

12. The dsRNA oligonucleotide of claim 10 wherein the dsRNA is a small interference RNA.
13. The siRNA oligonucleotide of claim 11 comprising 5-40 nucleotides in length.
14. The siRNA oligonucleotide of claim 12 comprising 15-25 nucleotides in length.
- 5 15. A dsRNA oligonucleotide comprising 5 to 40 nucleotides in length, wherein said oligonucleotide comprises a contiguous nucleotide sequence corresponding to a fragment selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO:20, which specifically hybridizes to an mRNA encoding human Tlr4.
- 10 16. An expression vector capable of expressing an oligonucleotide which is substantially complementary to a nucleic acid sequence encoding Tlr4 and which comprises at least an 8 nucleobase portion of any one of SEQ ID NOs:1-24.
17. The expression vector comprising the oligonucleotide according to any one of claims 3 to 9.
- 15 18. The expression vector comprising the oligonucleotide according to any one of claims 10 to 15.
19. An expression vector capable of expressing an oligonucleotide comprising at least an 8 nucleobase portion of anyone of SEQ ID NOs: 1-24, wherein said oligonucleotide inhibits expression of Tlr4.
- 20 20. A pharmaceutical composition comprising an oligonucleotide for inhibiting Toll-like receptor 4 (*tlr4*) gene expression, wherein said oligonucleotide comprises a contiguous nucleotide sequence selected from the group consisting of a fragment of the genomic sequence of Tlr4 or an mRNA transcribed therefrom.
- 25 21. A pharmaceutical composition wherein the oligonucleotide is selected from a group consisting of an antisense, a double-stranded RNA (dsRNA) and triple-stranded nucleic acids, enzymatic nucleic acid molecules, and aptamers
22. A pharmaceutical composition comprising as an active ingredient an oligonucleotide according to any one of claims 3-9.

23. A pharmaceutical composition comprising as an active ingredient an oligonucleotide according to any one of claims 10-15.
24. The pharmaceutical composition according to any one of claims 20-23, further comprising a pharmaceutically acceptable carrier, excipient or diluent.
- 5 25. A pharmaceutical composition comprising an expression vector and a pharmaceutically acceptable carrier, wherein said expression vector is capable of expressing an oligonucleotide which is substantially complementary to a nucleic acid sequence encoding Tlr4 and which comprises at least an 8 nucleobase portion of SEQ ID NOs:1-24.
- 10 26. A method of reducing the expression of Tlr4 in a cell comprising the step of contacting the cell with an antisense oligonucleotide comprising 5 to 50 nucleotides in length, wherein said oligonucleotide comprises a contiguous nucleotide sequence selected from the group consisting of a fragment of any one of SEQ ID NOs:1, 2 and 20 wherein said oligonucleotide reduces the expression of Tlr4 in said cell.
- 15 27. The method of claim 26 wherein the oligonucleotide is selected from any one of SEQ ID NOS:3-6, and 21-24, or an active fragment thereof.
28. A method of reducing the expression of Tlr4 in a cell comprising the step of contacting the cell with a dsRNA oligonucleotide having 5 to 40 nucleotides in length, wherein said oligonucleotide comprises a contiguous nucleotide sequence selected from the
- 20 group consisting of a fragment of any one of SEQ ID NOs:1, 2, and 20 wherein said oligonucleotide reduces the expression of Tlr4 in said cell.
29. The method of claim 28 wherein the oligonucleotide is selected from any one of SEQ ID NOs:8-19, or an active fragment thereof.
30. A method of preventing expression of Tlr4 by a cell comprising the step of introducing
- 25 into said cell a vector comprising the oligonucleotide of claim 2.
31. A method of preventing expression of Tlr4 by a cell comprising the step of introducing into said cell a vector comprising the oligonucleotide of claim 3.
32. The method of claim 30 wherein the oligonucleotide sequences attenuate Tlr4 expression within a cell ex-vivo.

33. The method of claim 30 wherein the oligonucleotide sequences attenuate expression of a target gene within a cell in-vivo.
34. A method for prevention or treatment of septic shock comprising the step of administering to a subject in need thereof a therapeutically effective amount of a pharmaceutical composition according to any one of claims 20-25.
35. The method of claim 32 wherein the subject is of mammalian origin.
36. The method of claim 32 wherein the subject is a human.
37. The method of claim 32 wherein the antisense is in an expression vector.
38. The method of claim 32 wherein the siRNA is in an expression vector.
39. A method for prevention or treatment of graft versus host disease comprising the step of administering to a patient in need thereof a therapeutically effective amount of a pharmaceutical composition according to any one of claims 20-25.
40. A method for treatment of inflammatory and autoimmune conditions related to Hsp60 comprising the step of administering to a patient in need thereof a therapeutically effective amount of a pharmaceutical composition according to any one of claims 20-25.
41. The method according to claim 41 wherein the inflammatory and autoimmune conditions are selected from the group of arthritis, type 1 diabetes, multiple sclerosis, inflammatory bowel disease, and atherosclerosis.
42. Use of an oligonucleotide molecule according to any one of claims 1-18 for preparation of a pharmaceutical composition.

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1  cctctcacc  tttagcccag  aactgctttg  aatacaccaa  ttgctgtggg  ggggtctgag
61  gaagagaaga  caccagtggc  tcagaaaactg  ctgggtcaga  cgggtgtagc  gagccaogca
121  ttccacagggc  cactgtgtgt  ccacagaaagca  gtgaggatga  tgccagggatg  atgtctgcct
181  cgggcctggc  tgggactctg  atcccagcca  tggccttcct  ctccctgctg  agaccagaaa
241  gctggggagcc  ctgctgggag  gtgggttocta  atattactta  tcaatgcatg  gagctgaatt
301  tctacaaaat  ccccgacaa  ctccccttct  caaccaagaa  cctggacctg  agctttaato
361  ccttgaggca  tttaggcagc  tatagcttct  tcagtttccc  agaactgcag  gtgctggatt
421  tatccagggtg  tgaatccag  acaattgaag  atggggcata  tcagagccta  agccacctct
481  ctaccttaat  attgacagga  aaccccatcc  agagtttagc  cctgggagcc  tttctgggac
541  tatcaagttt  acagaagctg  gtggctgtgg  agacaaatct  agcatctcta  gagaacttcc
601  ccattggaca  tctcaaaaact  ttgaaagaac  ttaatgtggc  tcacaatctt  atccaatctt
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Figure 1

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Figure 1 continued

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Figure 2

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Figure 2 continued

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Figure 3

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Figure 3 continued

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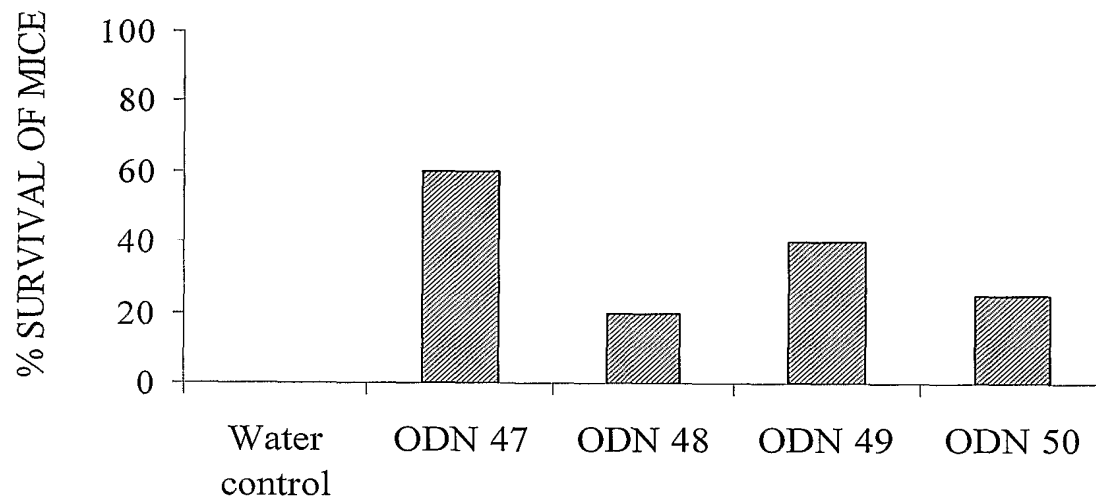
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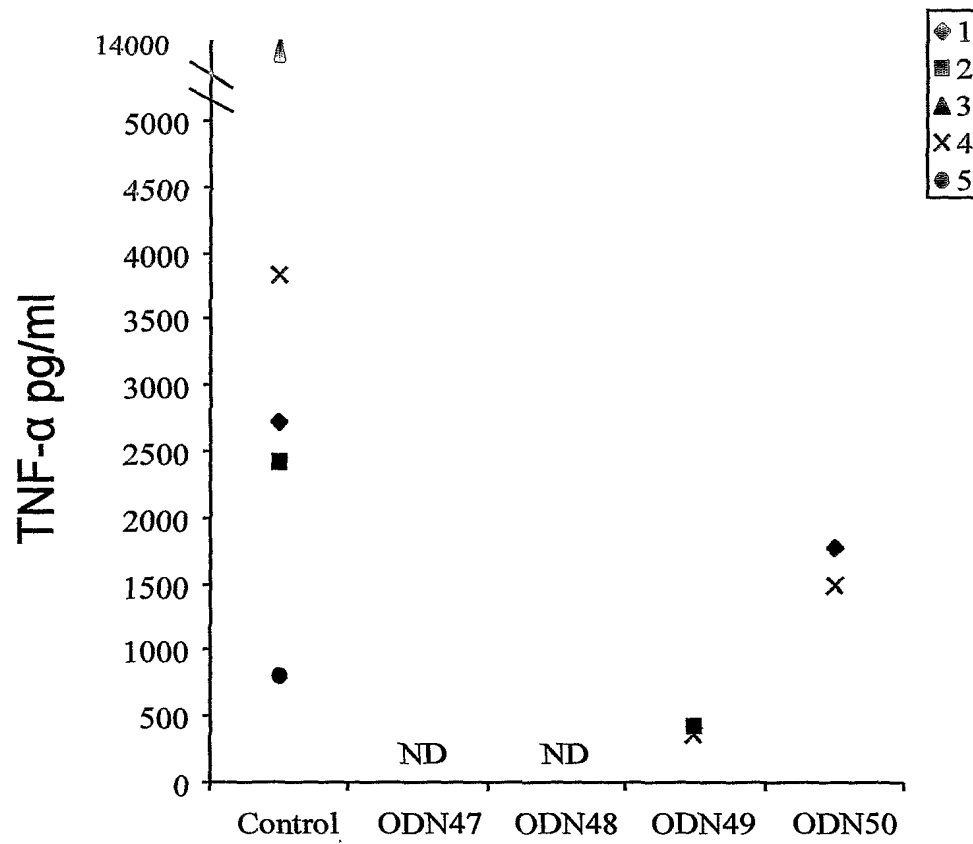
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Figure 3 continued

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**Figure 4**

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**Figure 5**

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(81) Designated States (*unless otherwise indicated, for every
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AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,
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ZW.

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ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE,
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ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: OLIGONUCLEOTIDES THAT BLOCK TOLL-LIKE RECEPTORS

(57) Abstract: Compositions and methods are provided for inhibiting the expression of Toll-like receptor-4 genes. Antisense and dsRNA oligonucleotides targeted to nucleic acids encoding toll-like-receptor-4 are preferred. Methods of using these oligonucleotides for inhibition of toll-like-receptor-4 expression, and thereby preventing the signaling of toll-like receptor-4 ligands and for treatment of diseases including septic shock, inflammatory and autoimmune diseases associated with toll-like receptor-4 ligands, are provided.



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INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL04/00349

A. CLASSIFICATION OF SUBJECT MATTER

IPC: C12Q 1/68(2006.01);C12P 19/34(2006.01);C12N 15/63(2006.01),15/58(2006.01);A01N 43/04(2006.01);C07H 21/02(2006.01),21/04(2006.01)

USPC: 435/6,91.1,91.31,455,458;514/44;536/23.1,24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.31, 455,458; 514/44; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
West, Dialog, STIC Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/77204 A1 (UNIVERSITY OF IOWA RES. FOUNDATION) 21 December 2000, page 4; page 17, second paragraph, pages 40-41; SEQ ID Nos. 43 and 67.	1-4 and 6-9
---		10, 12, 14-22, 25-26, 28, 30-32, 35-38
Y	US 6,506,559 B1 (FIRE et al.) 14 January 2003(01.14.2003), col. 1-8; 13; claims 1-8, 10-12, 15, 16, 21.	1-4, 6-10, 12, 14-22, 25, 26, 28, 30-32 and 35-38

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

04 January 2007 (04.01.2007)

Date of mailing of the international search report

29 JAN 2007

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Facsimile No. (571) 273-3201

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL04/00349

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 41 and 42
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Please See Continuation Sheet
3. ☒ Claims Nos.: 24,34,39 and 40
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-4,6-10,12,14-19,23,25,26,28,30-33 and 35-38

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees

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155561 24 April 2003 (24.04.2003) IL

(71) Applicant (for all designated States except US): **YEDA RESEARCH AND DEVELOPMENT CO. LTD.** [IL/IL]; Weizmann Institute of Science, P.O. Box 95, 76100 Rehovot (IL).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **COHEN, Irun, R.** [US/IL]; 11 Hankin Street, 76354 Rehovot (IL). **NUSS-BAUM, Gabriel** [US/IL]; 24/6 Bustenai Street, 93229 Jerusalem (IL).

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